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TITLE: HIV targeted ribozymes

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☐ 2. Document ID: US 5853716 A

Entry 2 of 6

File: USPT

Dec 29, 1998

US-PAT-NO: 5853716

DOCUMENT-IDENTIFIER: US 5853716 A

TITLE: Genetically engineered chimeric viruses for the treatment of diseases associated with viral transactivators

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☐ 3. Document ID: US 5830876 A

Entry 3 of 6

File: USPT

Nov 3, 1998

US-PAT-NO: 5830876

DOCUMENT-IDENTIFIER: US 5830876 A

TITLE: Genetic immunization

Full	Title	Citation	Front	Review	Classification	Date	Reference	Claims	KWC	Image
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☐ 4. Document ID: US 5817479 A

Entry 4 of 6

File: USPT

Oct 6, 1998

US-PAT-NO: 5817479

DOCUMENT-IDENTIFIER: US 5817479 A

TITLE: Human kinase homologs

Full	Title	Citation	Front	Review	Classification	Date	Reference	Claims	KWC	Image
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☐ 5. Document ID: US 5643761 A

Entry 5 of 6

File: USPT

Jul 1, 1997

US-PAT-NO: 5643761

DOCUMENT-IDENTIFIER: US 5643761 A

TITLE: Method for generating a subtracted cDNA library and uses of the generated library

Full	Title	Citation	Front	Review	Classification	Date	Reference	Claims	KWIC	Image
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☐ 6. Document ID: US 5527895 A

Entry 6 of 6

File: USPT

Jun 18, 1996

US-PAT-NO: 5527895

DOCUMENT-IDENTIFIER: US 5527895 A

TITLE: HIV targeted hairpin ribozymes

Full	Title	Citation	Front	Review	Classification	Date	Reference	Claims	KWIC	Image
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USPT	human near3 cell	23338	<u>L8</u>
USPT	human adj mammary adj cell	55	<u>L7</u>
USPT	promoter or enhancer	48589	<u>L6</u>
USPT	l1 or l2 or l3 or l4	1053	<u>L5</u>
USPT	mouse adj mammary adj tumor adj virus	585	<u>L4</u>
USPT	mmtv	620	<u>L3</u>
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AN 1991:672485 CAPLUS
DN 115:272485
TI Localization and induced expression of fusion genes in the rat lung
AU Hazinski, Thomas A.; Ladd, Patricia A.; DeMatteo, C. Anthony
CS Sch. Med., Vanderbilt Univ., Nashville, TN, 37232-2586, USA
SO Am. J. Respir. Cell Mol. Biol. (1991), 4(3), 206-9
CODEN: AJRBEL; ISSN: 1044-1549
DT Journal
LA English

L6 ANSWER 6 OF 6 CAPLUS COPYRIGHT 2000 ACS DUPLICATE 4
AN 1990:435810 CAPLUS
DN 113:35810
TI Glucocorticoids regulate expression of the v-mycOK10 oncogene in a murine
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AU Jacquemin-Sablon, Helene; Bogenberger, Jakob
CS Mol. Biol. Virol. Lab., Salk Inst., San Diego, CA, USA
SO Biochem. Int. (1990), 20(4), 669-79
CODEN: BIINDF; ISSN: 0158-5231
DT Journal
LA English

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Shin-Lin Chen

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Localization and Induced Expression of Fusion Genes in the Rat Lung

Thomas A. Hazinski, Patricia A. Ladd, and C. Anthony DeMatteo

Vanderbilt University School of Medicine, Department of Pediatrics, Nashville, Tennessee

Liposome-mediated gene transfer is useful for DNA transfection into cells in culture. We wondered whether this method could be used to introduce new DNA into the intact lung. Fusion genes containing either the Rous sarcoma virus (RSV) promoter or the mouse mammary tumor virus (MMTV) promoter (which contains glucocorticoid response elements) were linked to the bacterial gene chloramphenicol acetyltransferase (CAT), an enzyme not present in mammalian cells. Plasmids containing the RSV-CAT fusion gene were mixed with cationic liposomes (Lipofectin®; BRL, Inc., Grand Island, NY), and single doses were instilled into the cervical trachea of anesthetized rats. Control rats received either liposomes or plasmid. After 24, 48, and 72 h, lungs were perfused free of blood, homogenized, and analyzed for CAT enzyme activity. Liver and kidney tissue were also obtained. We found that rats given either intratracheal liposomes or plasmid had no detectable CAT activity. By contrast, 24 h after instillation of lipid:DNA complexes, lung CAT expression remained elevated for the next 48 h but was barely detectable in liver or kidney.

In another group of rats, MMTV-CAT:liposome complexes were instilled intratracheally and then the rats were injected with either dexamethasone or saline. We found that the dexamethasone-treated rats had a 5- to 10-fold higher level of lung CAT expression at 24 and 48 h than the saline-treated controls had; liver and kidney CAT levels were negligible in both groups. Dexamethasone treatment did not increase RSV-CAT expression, indicating that the dexamethasone effect on MMTV-CAT expression was related to the presence of the MMTV promoter. Using a third fusion gene construct, CMV- β -gal, we localized reporter gene expression to airway epithelium. We conclude that DNA:liposome complexes can be expressed and positively regulated in lung tissue *in vivo*. This new technique might be useful in the initial evaluation of therapeutic genes without resorting to retroviral vectors.

To be effective, human gene therapy will require methods to safely insert genes of therapeutic value into specific cells and to regulate their expression (1, 2). Gene expression has been conferred *in vivo* by *ex vivo* transfection and reintroduction of tissue onto organs (3-5) and by direct injection (6). However, these methods may not be useful for all situations. Recently, cationic lipids (7, 8) have been used to introduce reporter genes into liver (9), into porcine vascular tissue *in vivo* (10), and into the mouse (11), but specific cell targeting has been only partially successful. Because the lung has a large epithelial surface and is easily accessed via the trachea, we wondered if fusion genes could be inserted into the lung and regulated *in vivo*. Using three fusion gene constructs and cationic lipids, we found that intratracheally instilled genes could be expressed and positively regulated *in vivo* and that transient gene expression could be confined substantially to airway epithelium.

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Abbreviations: chloramphenicol acetyltransferase, CAT; cytomegalovirus, CMV; mouse mammary tumor virus, MMTV; Rous sarcoma virus, RSV. Am. J. Respir. Cell Mol. Biol. Vol. 4, pp. 206-209, 1991

Materials and Methods

Plasmid Preparation

Three plasmids derived from cloning vectors pBR322 or pUC, each containing a promoter sequence and one of two reporter genes, were obtained, amplified and verified using standard methods (12). We used: (1) RSV-CAT, the Rous sarcoma virus promoter linked to the reporter gene chloramphenicol acetyltransferase (CAT); (2) MMTV-CAT, the mouse mammary tumor virus (MMTV) promoter linked to CAT; and (3) CMV- β -gal, the CMV promoter linked to the *Escherichia coli* Lac-Z gene, which codes for the enzyme β -galactosidase. The activity of this enzyme can be visualized *in vivo* with the reagent X-gal ([5-bromo-4-chloro-3-indolyl β -D-Galactopyranoside] [Boehringer Mannheim Biochemicals, Indianapolis, IN]). The MMTV-CAT construct contains multiple copies of a glucocorticoid regulatory sequence that in cell culture studies results in enhanced transcription in the presence of glucocorticoids and the appropriate trans-acting elements (13). Each of the three plasmids also contained SV-40 splicing and polyadenylation sites to permit correct processing of RNA transcripts.

Fifteen minutes before tracheal instillation, 10 μ g plasmid DNA, 40 μ g lipid (Lipofectin®; GIBCO BRL, Inc., Grand Island, NY) and sterile water (to a total volume of 100 μ l) were gently mixed in a polystyrene tube and allowed to react

for 15 min at room temperature. Then, the lipid:DNA complex was aspirated into a 1-ml plastic syringe via a 27-gauge needle and injected into the trachea using the protocol described below.

Study Protocol

Adult pathogen-free rats of either sex were anesthetized with 2% halothane/98% oxygen via an open anesthesia circuit vented to the outside of the building. When anesthesia was induced, we lowered the halothane to 1.5%, placed the animal supine, shaved its neck, and cleaned the skin with antiseptic solution. Xylocaine, 1%, was infiltrated into the skin, and a 3-mm vertical incision was made. The cervical trachea was identified using blunt dissection, and 100 μ l of the lipid:DNA solution was injected directly into the trachea. We directly visualized the trachea in order to ensure that extratracheal extravasation of DNA did not occur. After injection of the lipid:DNA solution, the wound was sutured and anesthesia was stopped. The entire surgical procedure could be performed in less than 3 min. The animals were placed back into a cage with free access to food and water. The study protocol was approved by the Animal Care Committee at Vanderbilt University.

To determine if MMTV-CAT-treated rats would have enhanced CAT activity in the presence of glucocorticoids, rats were given either dexamethasone (4 mg/kg) or an equal volume of saline intramuscularly just prior to the administration of lipid:DNA complexes. To determine whether dexamethasone would nonspecifically enhance CAT expression, either dexamethasone or saline was also given to rats that received the RSV-CAT construct, which does not contain glucocorticoid regulatory sequences.

Every 24 h for 3 d after injection, rats were killed via CO₂ inhalation and lung, liver, and kidney tissue were obtained and frozen in liquid nitrogen until assayed for protein concentration and for CAT activity. In some animals, the pulmonary vessels were perfused until the lungs were blood-free. In other rats, bronchoalveolar lavage was also performed. These maneuvers were performed to determine whether intravascular cells or free alveolar cells were sites of CAT expression. The organs of rats who received CMV- β -gal were fixed in 1.25% glutaraldehyde prior to freezing (see below).

As controls, rats were given either naked DNA, lipid only, or DNA:lipid complexes that contained only plasmid vector without the relevant inserts.

Reporter Gene Assays

Frozen tissue was minced in a tissue grinder at 4° C, and cell protein lysates were prepared. The method of Nordeen and colleagues (14) was used to measure CAT activity, which is not normally present in mammalian cells (15). This assay uses tritiated acetate, cold acetate, and acetyl CoA synthase to generate tritium-labeled acetyl-coenzyme A; this latter compound acetylates chloramphenicol which appears in benzene-extractable form if CAT is present. The protein concentration of cell lysates was measured by the bicinchoninic acid method (16). CAT activity was expressed as cpm/mg protein, using sham-lipofected rat tissue to correct for background radioactivity. As additional controls, we tested for the possibility that rat tissue had endogenous deacetylation activity by adding 0.03 U bacterial CAT (Promega

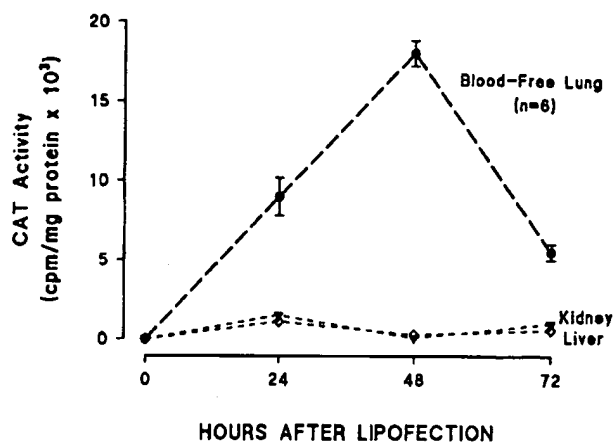


Figure 1. Organ distribution of CAT activity following intratracheal instillation of the RSV-CAT fusion gene complexed with cationic lipids. CAT activity was substantially confined to lung tissue. Each point represents the mean \pm 1 SD of six animals.

E105A; Promega, Madison, WI) to control rat tissue; we found that CAT could be detected regardless of the amount of lung, liver, or kidney lysates that were added. This indicated that endogenous deacetylation was not detectable in these tissues. To test for endogenous activity which converts labeled acetate to benzene-extractable forms, we also incubated cell lysates in reactions in which chloramphenicol was omitted from the buffer. We found no evidence of endogenous acetate metabolism which would overestimate our CAT results in lung, liver, or kidney. However, spleen cell lysates contained a large number of counts even in nontransfected animals. Therefore, spleen tissue was omitted from CAT analysis.

To identify the sites of β -galactosidase expression in the lung *in vivo*, we resected the lung *en bloc* and injected the trachea to an inflation pressure of 20 cm H₂O with cold 1.25% glutaraldehyde in 100 mM phosphate buffer, pH 7.4 (17). The lung, liver, and kidney were then submerged in cold glutaraldehyde for 15 min, and the tissues were frozen in liquid nitrogen and stored at -70° C. Nine-micron tissue

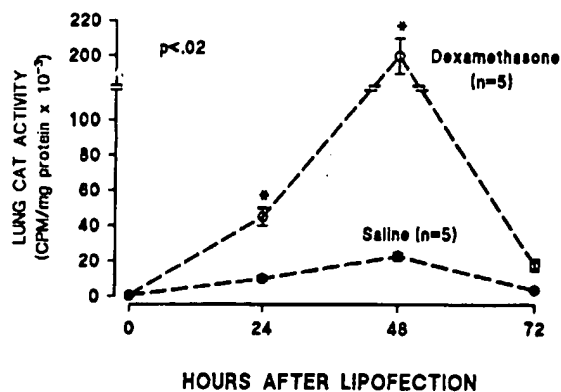


Figure 2. Effect of dexamethasone on CAT expression in MMTV-CAT fusion gene with cationic lipids. Dexamethasone treatment significantly stimulated CAT expression at 24, 48, and 72 h ($P < 0.02$). Each point represents the mean \pm SD of five animals.

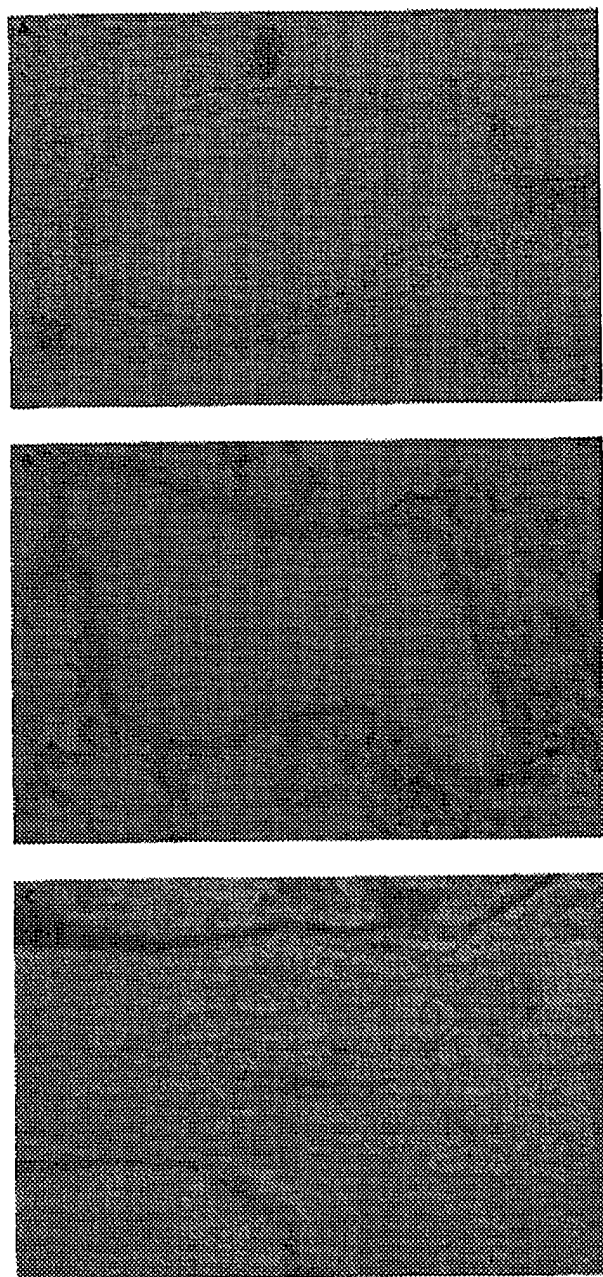


Figure 3. Localization of lung β -galactosidase activity in unstained frozen sections of sham-transfected (panel A) and CMV- β -gal-transfected (panel B) rats. Fusion genes were complexed with cationic lipids and delivered intratracheally. Blue color indicates β -galactosidase activity. Photomicrographs in panels A and B were taken at $250\times$ 48 h after transfection. Panel C depicts a $500\times$ view of the airway in panel B. When sections were examined under oil, both ciliated and nonciliated epithelial cells contained blue stain. In these sections, blue stain could be prevented if sections were incubated with 1,4-galactonolactone, confirming that X-gal metabolism was due to β -galactosidase activity. Blue color was confined to airway epithelium and was absent in liver and kidney. Scant non-epithelial staining is probably due to epithelial cells dislodged during tissue sectioning.

sections were cut in a cryostat, and the tissue ribbons were blotted onto gel-coated slides. The slides were allowed to dry briefly in air, then placed in X-gal staining solution which contained 150 mM 5-bromo-4-chloro-3-indolyl β -D-Galactopyranoside (Sigma B4252) and 1.0 mM spermidine in 100 mM phosphate buffer, pH 7.3. Just prior to use, freshly prepared ferroferricyanide was added to the staining solution (final concentration, 5 mM). No other counterstains were used. Slides were incubated at 37°C for 48 h, then dried, and permanently fixed with Permount[®]. Cover slips were applied, and slides were coded and examined for the presence of blue-stained cells which indicated that β -galactosidase activity was present. Endogenous β -galactosidase activity is not present in rat lung, liver, or kidney (16). Rat thymus, which is known to contain β -galactosidase activity, was used as a positive control. To further confirm that the blue staining indicated the presence of β -galactosidase, adjacent lung sections were incubated in 5 mM 1,4-galactonolactone, a specific inhibitor of β -galactosidase, which would ablate the staining in regions that stained blue with the X-gal reagent (18).

As controls, rats were given either naked DNA, lipid only, or DNA-lipid complexes that contained only plasmid vector without the relevant inserts.

Results

RSV-CAT Experiments

When rats were injected with either naked plasmid or lipid, CAT activity in lung, liver, and kidney was not present in these tissues. However, when RSV-CAT:lipid complexes were instilled, CAT activity was present in the lung for 72 h with a peak at 48 h (Figure 1). These results were the same when lung vessels were perfused or in lungs that were lavaged prior to CAT analysis, indicating that neither blood cells nor free alveolar cells were prominent sites of CAT expression. A small amount of CAT activity was also measured in liver and kidney (Figure 1).

MMTV-CAT Experiments

When rats were injected intratracheally with MMTV-CAT:lipid complexes, CAT activity was again present in the lung and this could be increased up to 10-fold at 48 h by dexamethasone pretreatment (Figure 2). Using this construct, CAT activity could not be demonstrated in liver or kidney. Dexamethasone treatment did not augment lung CAT activity in rats transfected with RSV-CAT plasmids; in fact, corticosteroid treatment reduced RSV-CAT expression by 40% at 48 h and 72 h (data not shown), suggesting that the dexamethasone effect on MMTV-CAT expression was due to the presence of the MMTV promoter.

CMV- β -gal Experiments

As shown in Figure 3, the airway epithelium was blue-stained in those rats that received intratracheal CMV- β -gal:lipid complexes. Blue staining was present in all airways examined in six rats at 48, 72, and 120 h after instillation. Tracheal, interstitial, alveolar, and endothelial cells were not stained. In 30 sections from five sham-transfected rats, only one section was found to be blue-stained: in this section, interstitial staining and airway staining was present. Other

than this one section, blue stain was absent from control rats. No blue staining could be detected in liver or kidney of any animal. In rats that received CMV- β -gal:lipid complexes, staining of airway epithelium could be ablated when the slides were preincubated with 1,4-galactonolactone.

Discussion

We found that intratracheal administration of three fusion genes using a lipid carrier resulted in detectable levels of fusion gene expression. One fusion gene construct, MMTV-CAT, could be positively regulated by a commonly used drug, dexamethasone; moreover, expression of the foreign gene linked to the CMV promoter could be substantially confined to lung epithelium *in vivo*. We did not examine other tissues, such as lymphoid tissue or muscle, and so we cannot exclude the possibility that other sites were transfected. To our knowledge, although others have introduced genes into the rodent lung (11), this is the first report to identify the sites of expression and to demonstrate positive regulation by an exogenous agent.

In tissue culture studies, it is possible to quantitate transfection efficiency but this is more difficult in *in vivo* studies. Our *in situ* identification of X-gal activity in airways of different size was surprisingly uniform. We estimate that at least 50% of the bronchial epithelium was stained at 48 h. This indicates that either CMV- β -gal expression is very efficient or that the enzyme or X-gal metabolite is stable intracellularly.

Although replication-defective virus-mediated gene transfer is highly efficient and holds great promise (19), these vectors can revert to replication-competent and can only infect dividing cells. By contrast, lipid carriers are not self-replicating, are nontoxic, and may be able to introduce genetic material, including RNA (20), into nondividing cells. The transient nature of gene expression may be overcome with multiple dosing and may in fact be a built-in safety factor in the initial testing of gene constructs for therapeutic value. Moreover, the transient nature of gene expression may be desirable if a transient effect is desired to augment lung defense against acute injury, such as radiation or oxygen exposure.

Many safety issues remain, and strategies to overcome the potential hazards of random insertion of fusion genes into the host cell genome need to be developed (21). However, the present study indicates that the transient introduction of genetic material can be accomplished in airway epithelium using simple techniques and that it is possible to positively regulate this expression. Improvements in liposome (22) and fusion gene construction, and the addition of proteins (23) or ligands for cell surface receptors to DNA: lipid complexes (24) may enable targeting of specific lung cells for gene therapy.

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HL-14214 (Newborn Lung SCOR). We thank Mark A. Magnuson, M.D. for providing plasmids and inserts and Elizabeth A. Perket, M.D. and Mahlan Johnson, M.D. for their assistance with the preparation and photography of tissue sections.

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Glucocorticoids regulate expression of the v-myc^{OK10} oncogene in a murine retroviral vector with chimeric MoMuLV-MMTV LTRs

Hélène JACQUEMIN-SABLON^{1,2} and Jakob BOGENBERGER¹

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SUMMARY

A murine retrovirus which expresses the v-myc^{OK10} oncogene under the control of the dexamethasone-regulatable mouse mammary tumor virus (MMTV) promoter has been constructed. In this vector, denoted pMImyc, the Moloney Murine leukemia virus (MoMuLV) sequences required for virus replication, integration and packaging were kept, while all the elements for transcription regulation were derived from the MMTV long terminal repeat (LTR). After transfection of NIH 3T3 fibroblasts with this construct, a cell line was isolated in which the level of v-myc RNAs were increased 60 fold by dexamethasone.

Kinetic studies showed that this induction can be maintained for up to 12 hours of hormone treatment. After infection with MoMuLV as a helper virus, and in the presence of dexamethasone, the production of pMImyc RNA, estimated by slot blot analysis, was equivalent to about 10³ viral particles/ml.

INTRODUCTION

Recent studies on the role of oncogenes in malignant transformation utilize transfection with plasmids in which oncogene expression is regulated by an inducible promoter. Such approaches have been reported with v-Ha-ras (1), N-ras (2), v-mos (3), v-src (4), polyomavirus middle T antigen (5) Ad12 E1A (6) and c-myc (7,8), all under regulation of the dexamethasone inducible promoter from the murine mammary tumor virus (MMTV). These plasmids are useful to study the effect of these oncogenes on various aspects of cell behavior, and to answer questions such as i) are there different thresholds in oncogene expression for the different phenotypic changes or other oncogene-mediated processes? and ii) is continuous expression of the oncogene required for the maintenance of the phenotypic changes observed?. These studies however are limited by the difficulty in transfecting foreign DNA into many cell types, especially hematopoietic cells. Retroviral vectors

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offer unique advantages since, in principle, they can be used to introduce an intact single copy of a gene into most mammalian cell types, often at efficiencies approaching 100%.

We wished therefore to obtain a retroviral vector in which the *myc* gene is under the control of the MMTV promoter. Disadvantages of using MMTV as a tool for gene transfer are the limited infection spectrum displayed by MMTV *in vivo* and *in vitro* (9), and the low titer at which it is produced (10,11). The most efficient murine retroviral vectors are MoMuLV derived vectors. Vennström et al. (12) have constructed a MoMuLV and Ha-MSV based retroviral vector denoted MMCV which expresses the *v-myc*^{OK10} oncogene. This led us to retain in our construct the general organisation of the MMCV vector which was made glucocorticoid inducible by modifying the LTRs: The MoMuLV and Ha-MSV promoter and enhancers were replaced by the glucocorticoid regulatory sequences and the promoter from MMTV (13,14,15).

We describe here the construction of this vector, denoted pMImyc. After transfection of NIH3T3 cells with pMImyc plasmid DNA, one cell line, M2, was isolated in which *v-myc* expression was increased about 60 fold after induction by dexamethasone. The induction by dexamethasone lasted for 12 hours. Infection of the cell line M2 with MoMuLV as a helper virus led to the production of pMImyc virion RNA. The production of virion RNA was inducible by dexamethasone.

MATERIAL AND METHODS

Transfection and selection

The NIH 3T3 cell line and the MMCV producer cell line, C5G, were provided by M. Vogt and B. Vennström, and cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. The MoMuLV producer cell line was provided by D. Dumenil. Cells were transfected with calcium-phosphate precipitated plasmid and carrier DNAs by a standard procedure (16). After 4h, transfection medium was replaced by fresh medium, and the following day, dishes were split 1/5. Selection with 400 µg/ml G418 (Gibco) was begun 2 days later and maintained with two weekly changes of medium.

Northern blot analysis Cells were grown to confluency in 100 mm culture dishes, and then maintained for 48 h in culture medium supplemented with 0.5% serum. Dexamethasone was then added. RNA was extracted by the guanidine thiocyanate technique (17) and 4 µg of total RNA per lane was electrophoresed through 1.2% agarose gels containing formaldehyde. Transfer onto an uncharged nylon membrane (Amersham hybond N) and hybridization were performed as previously described (18). RNA probes labelled with ³²P-UTP at a specific activity of 5 X 10⁸ cpm/µg were obtained by *in vitro* run off transcription of bluescript vectors, using phage specific RNA polymerases. For detection of *v-myc* expression, we used a pBT-*v-myc* plasmid which contains a 0.86 Kb *SalI*-*Bam*H1 fragment from *v-myc*^{OK10} exon 3 cloned in bluescript (Stratagene, La Jolla USA). The pBT-actin plasmid contains a *TaqI*-*Pst*I

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fragment of a murine cDNA cloned in bluescript. The pBT-gag plasmid contains a 1.3 Kb PvuII-EcoRI fragment from the N2 retroviral vector (14) containing R and U5 from the 5' LTR and gag sequences cloned in bluescript.

Detection of viral RNA

Cell culture supernatants (2.5 ml per 10 cm plate) were passed through a 0.45 μ m filter, loaded on a 3 ml glycerol cushion (20% glycerol in 0.05M Tris pH7.4, 0.1 MKCl), and pelleted at 100,000 g for 1.5 h at 4°C. For slot blot analysis, the pellet was resuspended in 200 μ l of lysis buffer (0.01 M Tris, pH7.4, 0.001 M EDTA, 0.05 M NaCl and 0.2% SDS) containing 100 μ g/ml proteinase K, and incubated for 1 hour at 50°C. Samples were then extracted with phenol and chloroform, and precipitated with ethanol. After denaturation in formaldehyde they were applied to a slot blot at different concentrations. For Northern blot analysis, the pellet was resuspended in 2 ml of guanidium thiocyanate solution and centrifuged on a 5.7M CsCl cushion, at 175000 Xg for 20 hours at 20°C in a SW60 rotor. RNA was resuspended in 6 μ l H₂O.

RESULTS AND DISCUSSION

a) Construction of the Chimeric LTR

A chimeric MoMuLV-MMTV LTR was constructed, containing mostly MMTV sequences. This LTR was then used to construct an MoMuLV based retroviral vector, with the MMTV steroid inducible promoter. Only very small portions of the 5' left end of U3 and of the 3' right end of U5 from the MoMuLV LTR were kept in order to retain the inverted repeats involved in the process of integration (Fig 1,A). The 560 bp Sau3A/MstII MMTV fragment in the chimeric LTR, contains the four elements within the glucocorticoid regulatory region of MMTV DNA involved in the stimulation of transcription by the hormone and the promoter which has been precisely located between position -200 and -50 (14). In MMTV, the poly(A) addition signal is located in U3 at position -76, while in MoMuLV it is located in R. It was therefore necessary to keep the MMTV R sequences to avoid a redundancy of these sequences in the final construct. This led us to generate a proviral DNA organisation in which both the 5' and 3' LTRs were modified. As cloning vehicles to construct the pMU35 plasmid (Fig.1A), we used the MoMuLV-LTR subclone pMLV C/R/B (19), and an MMTV LTR subclone (20).

b) Construction of the pMImyc retroviral vector

The plasmid pMU35 containing the chimeric LTR was used to construct the retroviral pMImyc vector containing v-myc sequences. This vector should place the expression of the viral RNAs under the control of steroid hormones. As shown in Fig.1B, the pMImyc retroviral vector was obtained in a single step by ligating together the two chimeric LTRs and a fragment from the MMCV vector containing the v-myc^{OK10} sequences. The pMImyc vector contains all the MoMuLV sequences necessary for replication and packaging of viral RNA into viral particles, and the transcription should be steroid-dependent since all the regulatory elements are derived from the MMTV LTR.

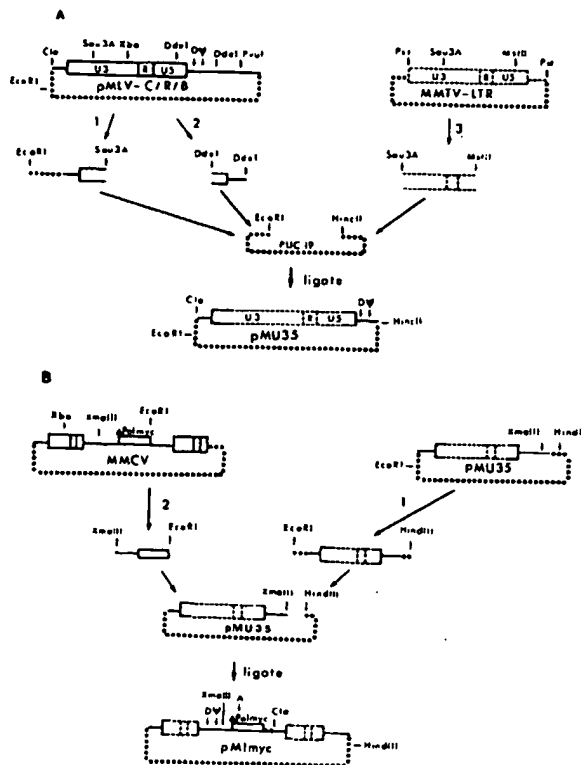


Figure 1A - Construction of the pMU35 plasmid

PMLVC/R/B was digested by EcoRI and Xba and the 0.46 Kb fragment containing MoMuLV U3 sequences was then digested by Sau 3A to give the 0.26 Kb fragment EcoRI-Sau3A (Fig.1A,1). MMTV-LTR plasmid was digested by Pst and MstII. The 1.3 Kb fragment containing the MMTV LTR was then partially digested by Sau3A and the 0.58 Kb Sau3A-MstII fragment was isolated (Fig.1A,3). pMLV C/R/B was also digested with Xba and PvuI. The 0.57 kb fragment was then digested by DdeI, the ends were filled with Klenow DNA polymerase I, and the 0.295 kb DdeI-DdeI fragment was isolated (Fig.1A,2). The DdeI-DdeI fragment containing MoMuLV U5 and 5' non coding region sequences, the pMLV C/R/B EcoRI-Sau3A fragment and the MMTV-LTR Sau3A-MstII fragment were ligated together in PUC 19 plasmid digested with EcoRI and HindIII to give the pMU35 plasmid. ----- MoMuLV sequences, - - - MMTV sequences, plasmid sequences

Figure 1B - Construction of the pMImyc plasmid

The pMU35 plasmid was digested with EcoRI and HindIII, and the 1.1 Kb EcoRI-HindIII fragment, containing the chimeric LTR was isolated (Fig.1B,1). The MMCV plasmid (20) was digested with Xba and EcoRI. The 4.4 Kb Xba-EcoRI fragment was then partially digested with XmaIII and the 4.1 Kb XmaIII-EcoRI fragment was isolated (Fig.1B,2). This fragment, containing 5' non coding region and part of gag sequence from MoMuLV, and pol-myc from OK10 virus, was ligated into the XmaIII-HindIII pMU35 vector together with the pMU35 EcoRI-HindIII fragment generating the pMImyc plasmid. ----- MoMuLV sequences, - - - MMTV sequences, plasmid sequences.

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The established mouse fibroblast cell line NIH 3T3 used as a recipient for DNA transfection showed a flat morphology and anchorage-dependence for growth. pMImyc and pSV2-neo plasmid DNAs were mixed in a ratio of 20 to 1 and transfected into NIH 3T3 cells. Cells were then selected for resistance to G418 in the absence of dexamethasone. Twenty individual colonies were established as cell lines and tested by Northern blotting for v-myc expression in the presence or absence of dexamethasone. Two clones had a v-myc inducible expression; the most inducible, clone M2 was analysed further.

We studied by Northern blot analysis the steady state levels of v-myc mRNAs after steroid stimulation. Dexamethasone was added to M2 cells previously grown to confluency and kept for 48 hours in 0.5% serum. As shown on Fig.2A, mRNAs of 4 different lengths hybridize with the v-myc probe. The level of 3 of these mRNAs increases after incubation with dexamethasone, while one mRNA of 3 kb shows a constitutive expression. The lengths of the 3 inducible mRNAs are as expected for a correct LTR initiation. The 5 kb mRNA represents the genomic pMImyc RNA, the 2.6 kb mRNA the subgenomic pMImyc RNA, and the 4 kb mRNA corresponds to the third (4.7 kb) mRNA of MMCV of unknown structure. The constitutive mRNA of 3 kb, which hybridizes with the v-myc probe, arose probably from a pMImyc rearrangement at the time of transfection.

The expression of the three viral v-myc RNAs in absence of hormone is barely detectable (Fig.2A, lane 1), even after prolonged exposure of the filter. They are detectable 1 hour after addition of dexamethasone and their level reaches a maximum 6 to 8 hours after hormone treatment (Fig.2A,lanes 2 to 6). Densitometric analysis indicates that the induced levels of the three v-myc RNAs are approximatively 60 times higher than in the uninduced M2 cells (Fig.2B). After 24 hours, a sharp decrease of the 3 viral RNA levels can be seen; the very low level of these RNAs persists after 48 h and 72 h of exposure to glucocorticoid hormone. As a control, it is shown that dexamethasone has no effect on β actin expression (Fig.2A, lower part).

The results of the induction of *v-myc* RNAs as a function of dexamethasone concentration is shown in Fig.3A. Induction is detected with 10^{-8} M dexamethasone, (Fig.3A, lane 3), and reaches a maximum with 5×10^{-7} M to 10^{-6} M (Fig.5A, lanes 6 and 7). The half maximal dose for induction, around 5×10^{-8} M, indicates a response to physiological levels of hormone. Densitometric analysis shows a 60 fold induction of the 5.1 Kb *v-myc* RNA (Fig.3B). These characteristics of *v-myc* expression (kinetics

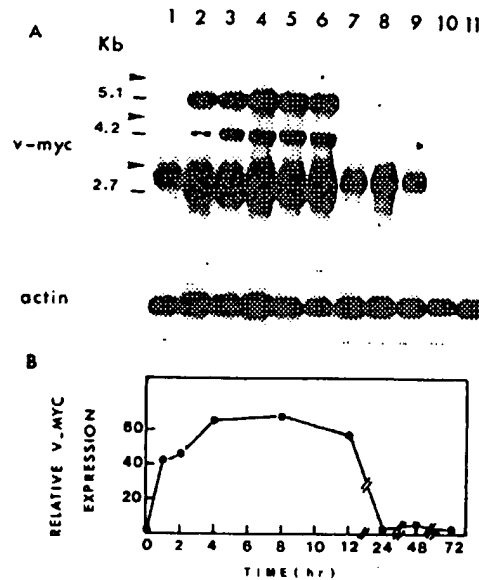


Figure 2 - Kinetics of induction of v-myc RNAs by dexamethasone in M2 cells. (A) Northern blot analysis of v-myc RNAs. Cells previously maintained quiescent as described under Methods were treated with 5×10^{-7} M dexamethasone for the following length of time; 0 min. (lane 1), 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), 8 h (lane 5), 12 h (lane 6), 24 h (lane 7), 48 h (lane 8), and 72 h (lane 9). Lane 10 NIH 3T3 cells in the absence of dexamethasone, lane 11, NIH 3T3 treated for 8 hours. 4 μ g of total RNA were loaded on agarose-formaldehyde gel, and the filter was hybridized to v-myc probe (upper part) or β actin probe (lower part). (B) Kinetics of induction of v-myc 5.1 kb RNA. The relative concentration of v-myc RNA was determined by densitometric scanning of the autoradiogram shown in Panel A as described in the text.

and dependence on hormone concentration) are in agreement with an induction mediated through the interaction between dexamethasone and its receptor.

f) Rescue of pMImyc

In order to produce recombinant pMImyc virus, M2 cells were infected with MoMuLV as a helper. Helper infected M2 cells were then treated with dexamethasone under optimal conditions for induction of the viral 5.1 kb RNA transcript (quiescent cells and 5.10^{-7} M dexamethasone). Cells were exposed for a longer period (15 hours) to the hormone to allow virus encapsidation and production. Packaging of pMImyc into virions was demonstrated by dot blot analysis of RNA after purification of the putative virion containing fraction from the medium of M2 cells (21). As shown on Fig.4A, RNAs present in the supernatants of M2 and C5G cells hybridize with the v-myc probe (lanes 1, 2 and 5) and the intensity of the band depends on the RNA concentration. Using the same conditions, we did not detect any signal in the super-

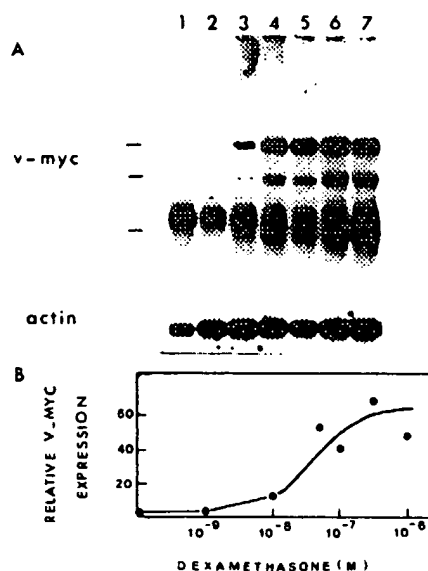


Figure 3 - (A) Northern blot analysis of v-myc RNAs as a function of dexamethasone concentration in M2 cells. Cells previously maintained quiescent were treated for 8 h with dexamethasone at different concentrations : 0 (lane 1), 10⁻⁹M (lane 2), 5 X 10⁻⁸M (lane 3), 10⁻⁸M (lane 4), 10⁻⁷M (lane 5), 5 X 10⁻⁷M (lane 6), 10⁻⁶M (lane 7). Upper part : v-myc probe, lower part, β actin probe. **(B)** Induction of v-myc 5.1 kb RNA as a function of dexamethasone concentration. The relative concentration of v-myc RNA was determined by densitometric scanning of the autoradiogram shown in Panel A as described in the text.

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lls were infected with :d with dexamethasone A transcript (quiescent nger period (15 hours) kaging of pMImyc into r purification of the lls (21). As shown on s hybridize with the v- depends on the RNA ny signal in the super-

natants of NIH 3T3 cells, or of a cell clone producing MoMuLV (lanes 3 and 4). In a parallel slot blot, the same RNA samples were hybridized with an antisense gag-LTR probe (Fig.4B). This probe detects MoMuLV sequences present both in the pMImyc vector and in the MoMuLV helper. By comparing the intensities of the signals, the approximate titer of pMImyc virus is about 10³ viral particles/ml. To determine if the RNA present in the supernatant of M2 cells was of the expected size, we performed a Northern blot analysis of the viral RNA. The RNA recovered from the supernatant of M2 cells hybridizes with the v-myc probe and is of the expected size (5.1 kb). It migrates to the same position in the gel as the larger v-myc RNA present in M2 cells (Fig.4C, lanes 3 and 4). The two other subgenomic v-myc RNAs (4.2 and 2.7 kb) are not detected, since they are not packaged. A 3.6 fold increase in the amount of viral RNA is observed in the supernatant of M2 cells treated with dexamethasone (lane 4), while there is no detectable change in the amount of viral RNA in the supernatant of C5G cells (lanes 1 and 2).

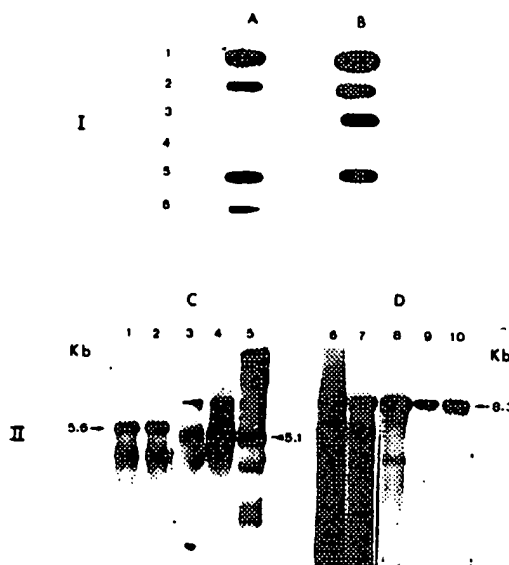


Figure 4 - I) RNA slot blot analysis of cell supernatants - 4 X 10 cm plates of quiescent cells were treated for 16 hours with 5×10^{-7} M dexamethasone (M2 cells), or were kept without hormone (C5G cells). Viral RNA, concentrated and purified as described under methods was resuspended in 20 μ l H₂O. 5 μ l of the concentrated supernatants were applied to a slot blot and hybridized with a v-myc probe (A) or to gag-LTR probe (B). The following supernatants were applied: lane 1: M2 cells, undiluted; lane 2: M2 cells 1/10 diluted; lane 3: MoMuLV producer cell clone, undiluted; lane 4: NIH3T3 cells, undiluted; lane 5: C5G cells, 1/1000 diluted (A) or undiluted (B); lane 6: C5G cells, 1/10000 diluted (A).

II) Northern blot analysis of viral RNA in cell supernatants. Cell supernatants from 10 X 10 cm plates of quiescent M2 cells untreated or treated with 5×10^{-7} M dexamethasone for 16 hours, or from 1 X 10 cm plate of C5G cells, were concentrated and RNA extracted as described under methods. 5 μ l of RNA (C) or 1 μ l (D) was loaded on agarose-formaldehyde gel, and the filter was hybridized with a v-myc probe (C) or a gag-LTR probe (D). The following supernatants were loaded: lanes 1 and 6: C5G cells, no dexamethasone; lanes 2 and 7: C5G cells treated with dexamethasone; lanes 3 and 8: M2 cells, no dexamethasone; lane 4 and 9: M2 cells treated with dexamethasone; lane 5 and 10: 4 μ g of total RNA from M2 cells treated for 8 hours with 5×10^{-7} M dexamethasone.

Autoradiography for slot blot and Northern blot analysis was for one hour (gag-LTR probe) or 24 hours (v-myc probe).

DISCUSSION

In this paper, we report the construction of a retroviral vector, pMImyc, in which the v-myc^{OK10} oncogene expression is under the control of the glucocorticoid inducible MMTV promoter. This vector was derived from the MoMuLV based MMCV described by Vennström et al. (12), which expresses v-myc via a subgenomic RNA. In the pMImyc vector, both 5' and 3' LTRs are chimeric MoMuLV-MMTV LTRs, in which all transcriptional regulatory elements are from MMTV so that the virion RNA and the subgenomic v-myc RNA expression can be regulated by glucocorticoids. This vector contains also all the sequences required for MoMuLV replication, packaging and integration so that infectious virus can be produced.

After transfection of NIH 3T3 cells with pMImyc plasmid DNA, we have isolated one cell line, M2, in which the level of the three v-myc RNAs can be induced by dexamethasone up to 60 fold above the basal level. This high inducibility might result from the fact that both enhancers and promoter in pMImyc are from MMTV (14), and that both 5' and 3' LTRs contain the MMTV HRE sequences (24). Kinetic studies showed that the v-myc RNAs, including the genomic viral transcript, reached a steady state level by 4-8 hours after hormone addition. This level persisted until 12 hours after the addition of dexamethasone and then sharply decreased. Similar kinetics of MMTV promoter induction by dexamethasone have been recently reported by Owen and Ostrowski (22), using a MMTV-v-ras construct transfected into NIH 3T3 cells. As discussed by Jaggi et al. (23), this transitory induction of the MMTV promoter could depend on the inserted gene.

M2 cells previously infected with MoMuLV as a helper virus were tested for pMImyc virus production. Since MMCV efficiently confers to fibroblasts anchorage-independent growth (12), it was postulated that NIH3T3 infected with pMImyc virus would grow in methyl cellulose in the presence of dexamethasone. However, we could not use this assay to titer pMImyc virus, since only cells expressing v-myc from rearranged viral sequences were recovered from methylcellulose. It is likely therefore that the transitory induction of v-myc expression in the pMImyc vector after hormone treatment does not allow anchorage independent growth as observed with MMCV which displays a strong and constitutive v-myc expression. Since the pMImyc construct does not contain a selectable gene to eliminate promoter interferences, virus production was detected using a biochemical assay. Slot blot and Northern blot analysis of virion RNA present in cell supernatants demonstrate that M2 cells, together with MoMuLV helper, produce pMImyc virus. The pMImyc virus production is increased at least 3.6 fold after treatment with dexamethasone, and the estimated titer is around 10^3 particles/ml. As compared to the glucocorticoid-responsive MoMuLV

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described by Overhauser and Fan (24), the pMIMyc virus is produced at a lower titer, but cytoplasmic RNA induction is much higher. The main difference lies in the presence of both MoMuLV and MMTV enhancers in Overhauser and Fan vector which probably allows for a higher virus production, but also accounts for a high basal activity of the promoter in absence of dexamethasone.

The vector system described here allows a very high inducibility of myc transcription by steroids with an almost undetectable background transcription in the absence of steroids. As the transcription level is greatly reduced after 24 hours of steroid induction, this vector system is especially useful to isolate genes that are potential targets for myc activity.

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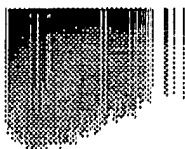
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L2 0 S HUMAN(W)MMTV(W) PROMOTER
L3 827 S MMTV(W) (PROMOTER OR REGULATORY(W)SEQUENCE)
L4 12821 S RETROVIRAL(W)VECTOR
L5 15 S L3 AND L4
L6 6 DUP REM L5 (9 DUPLICATES REMOVED)
L7 128 S WAP(W) (PROMOTER OR REGULATORY(W)SEQUENCE)
L8 429 S HUMAN(W)MAMMARY(W)CELL
L9 1 S L7 AND L8

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L6 ANSWER 1 OF 6 CAPLUS COPYRIGHT 2000 ACS DUPLICATE 1
AN 1998:52972 CAPLUS
DN 128:201466
TI Construction and characterization of a hybrid mouse mammary tumor
virus/murine leukemia virus-based **retroviral vector**
AU Saller, Robert M.; Ozturk, Feride; Salmons, Brian; Gunzburg, Walter H.
CS Inst. Molekulare Virol., GSF-Forschungszentrum Umwelt Gesundheit, Munich,
D-85758, Germany
SO J. Virol. (1998), 72(2), 1699-1703
CODEN: JOVIAM; ISSN: 0022-538X
PB American Society for Microbiology
DT Journal
LA English

L6 ANSWER 2 OF 6 CAPLUS COPYRIGHT 2000 ACS DUPLICATE 2
AN 1998:223850 CAPLUS
DN 129:228
TI Antisense c-myc **retroviral vector** suppresses
established human prostate cancer
AU Steiner, Mitchell S.; Anthony, Catherine T.; Lu, Yi; Holt, Jeffrey T.
CS Departments of Urology and Cell Biology, Vanderbilt University School of
Medicine, Nashville, TN, 37235, USA
SO Hum. Gene Ther. (1998), 9(5), 747-755
CODEN: HGTHE3; ISSN: 1043-0342
PB Mary Ann Liebert, Inc.
DT Journal
LA English

L6 ANSWER 3 OF 6 CAPLUS COPYRIGHT 2000 ACS
AN 1998:413000 CAPLUS
DN 129:132177
TI Inducible gene expression from promoter conversion **retroviral
vectors**
AU Salmons, Brian; Saller, Robert M.; Mrochen, Stefan; Ozturk, Feride;
Klein, Dieter; Gunzburg, Walter H.
CS Bavarian Nordic Research Institute, Munich, D-80807, Germany
SO Nucleic Acids Symp. Ser. (1998), 38(Advances in Gene Technology:
Molecular

Biology in the Conquest of Disease), 179-180
 CODEN: NACSD8; ISSN: 0261-3166
 PB Oxford University Press
 DT Journal
 LA English

L6 ANSWER 4 OF 6 CAPLUS COPYRIGHT 2000 ACS
 AN 1997:265594 CAPLUS
 DN 126:247559
 TI Rodent whey acid protein (WAP) or mouse mammary tumor virus (MMTV)
) **regulatory sequences** for targeted expression of
 heterologous genes in human mammary cells and applications in carcinoma
 gene therapy
 IN Guenzburg, Walter H.; Saller, Robert Michael; Salmons, Brian
 PA Bavarian Nordic Research Institute A/s, Den.; Gsf - Forschungszentrum
 Fuer Umwelt Und Gesundheit; Guenzburg, Walter H.; Saller, Robert Michael;
 Salmons, Brian
 SO PCT Int. Appl., 46 pp.
 CODEN: PIXXD2
 DT Patent
 LA English
 FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9709440	A1	19970313	WO 1996-EP3922	19960906
W:	AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM			
RW:	KE, LS, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM			
AU 9669876	A1	19970327	AU 1996-69876	19960906
EP 848757	A1	19980624	EP 1996-931040	19960906
R:	AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI			
JP 11511979	T2	19991019	JP 1996-510876	19960906
PRAI DK 1995-976		19950906		
WO 1996-EP3922		19960906		

L6 ANSWER 5 OF 6 CAPLUS COPYRIGHT 2000 ACS DUPLICATE 3
 AN 1991:672485 CAPLUS
 DN 115:272485
 TI Localization and induced expression of fusion genes in the rat lung
 AU Hazinski, Thomas A.; Ladd, Patricia A.; DeMatteo, C. Anthony
 CS Sch. Med., Vanderbilt Univ., Nashville, TN, 37232-2586, USA
 SO Am. J. Respir. Cell Mol. Biol. (1991), 4(3), 206-9
 CODEN: AJRBEL; ISSN: 1044-1549
 DT Journal
 LA English

L6 ANSWER 6 OF 6 CAPLUS COPYRIGHT 2000 ACS DUPLICATE 4
 AN 1990:435810 CAPLUS
 DN 113:35810
 TI Glucocorticoids regulate expression of the v-mycOK10 oncogene in a murine
retroviral vector with chimeric MoMuLV-MMTV LTRs
 AU Jacquemin-Sablon, Helene; Bogenberger, Jakob
 CS Mol. Biol. Virol. Lab., Salk Inst., San Diego, CA, USA
 SO Biochem. Int. (1990), 20(4), 669-79
 CODEN: BIINDF; ISSN: 0158-5231
 DT Journal
 LA English

=> d bib 19

L9 ANSWER 1 OF 1 CAPLUS COPYRIGHT 2000 ACS
AN 1997:265594 CAPLUS
DN 126:247559
TI Rodent whey acid protein (WAP) or mouse mammary tumor virus (MMTV)
regulatory sequences for targeted expression of heterologous genes in
human mammary cells and applications in
carcinoma gene therapy
IN Guenzburg, Walter H.; Saller, Robert Michael; Salmons, Brian
PA Bavarian Nordic Research Institute A/s, Den.; Gsf - Forschungszentrum
Fuer Umwelt Und Gesundheit; Guenzburg, Walter H.; Saller, Robert Michael;
Salmons, Brian
SO PCT Int. Appl., 46 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
	-----	---	-----	-----	-----
PI	WO 9709440	A1	19970313	WO 1996-EP3922	19960906
	W: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
	RW: KE, LS, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM				
	AU 9669876	A1	19970327	AU 1996-69876	19960906
	EP 848757	A1	19980624	EP 1996-931040	19960906
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI				
	JP 11511979	T2	19991019	JP 1996-510876	19960906
PRAI	DK 1995-976		19950906		
	WO 1996-EP3922		19960906		

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:33:

ATTACGCGTT TATTACAGAA TGGAAAACAG ATGGCAGGTG

4 0

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ATTACGCGTT ATTGCAGAA TCTTATTATG GC

3 2

We claim:

1. A method of immunizing an individual against a pathogen comprising the steps of:
 - injecting into skeletal muscle tissue of said individual at a site on said individual's body, bupivacaine and a DNA molecule that comprises a DNA sequence that encodes an antigen from said pathogen, said DNA sequence operatively linked to regulatory sequences which control the expression of said DNA sequence;
 - wherein said DNA molecule is taken up by cells in said skeletal muscle tissue, said DNA sequence is expressed in said cells and a protective immune response is generated against said antigen.
2. The method of claim 1 wherein said pathogen is an intracellular pathogen.
3. The method of claim 1 wherein said pathogen is a virus.
4. The method of claim 1 wherein said pathogen is a virus selected from the group consisting of: human T cell leukemia virus, HTLV; influenza virus; hepatitis A virus; hepatitis B virus; hepatitis C virus; human papilloma virus, HPV; Herpes simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus, CMV; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.
5. The method of claim 1 wherein said pathogen is Herpes simplex 2 virus, HSV2.
6. The method of claim 1 wherein said pathogen is Hepatitis B virus, HBV.
7. The method of claim 1 wherein said pathogen is human T cell leukemia virus, HTLV.
8. A method of treating an individual who has a hyperproliferative disease comprising:
 - injecting into skeletal muscle tissue of said individual at a site on said individual's body, bupivacaine and a DNA molecule that comprises a DNA sequence that encodes a hyperproliferative disease-associated protein operatively linked to regulatory sequences;
 - wherein said DNA molecule is taken up by cells in said skeletal muscle tissue, said DNA sequence is expressed in said cells, and a therapeutically effective immune response is generated against said hyperproliferative disease-associated protein.
9. The method of claim 8 wherein said hyperproliferative disease is cancer.
10. The method of claim 8 wherein said hyperproliferative disease is a lymphoma.
11. The method of claim 8 wherein said hyperproliferative disease is T cell lymphoma and said hyperproliferative disease-associated protein is a T cell antigen.
12. The method of claim 8 wherein said hyperproliferative disease is T cell lymphoma and said DNA sequence encodes a variable region of a T cell receptor.
13. The method of claim 8 wherein said hyperproliferative disease is a melanoma.
14. A method of treating an individual who is infected by a pathogen comprising:
 - injecting into skeletal muscle tissue of said individual at a site on said individual's body, bupivacaine and a DNA molecule that comprises a DNA sequence that encodes an antigen from said pathogen, said DNA sequence operatively linked to regulatory sequences which control the expression of said DNA sequence;
 - wherein said DNA molecule is taken up by cells in said skeletal muscle tissue, said DNA sequence is expressed in said cells and a therapeutically effective immune response is generated.
15. The method of claim 14 wherein said pathogen is an intracellular pathogen.
16. The method of claim 14 wherein said pathogen is a virus.
17. The method of claim 14 wherein said pathogen is human immunodeficiency virus HIV.

* * * * *

=> d his

(FILE 'HOME' ENTERED AT 12:00:12 ON 17 FEB 2000)

FILE 'MEDLINE, CAPLUS, BIOSIS, SCISEARCH' ENTERED AT 12:00:22 ON 17 FEB 2000

L1 974 S WHEY(W)ACID(W)PROTEIN OR WAP
L2 8662 S MOUSE(W)MAMMARY(W)TUMOR(W)VIRUS OR MMTV
L3 9589 S L1 OR L2
L4 378235 S PROMOTER OR ENHANCER OR REGULATORY(W)ELEMENT
L5 2916 S L3 AND L4
L6 6514 S HUMAN(5A)MAMMARY(3A)CELL
L7 56047 S RETROVIRAL(W)VECTOR OR RETROVIRUS
L8 117 S L7 AND L5
L9 3 S L8 AND L6
L10 802392 S HUMAN(5A)CELL
L11 23 S L10 AND L8
L12 3 DUP REM L9 (0 DUPLICATES REMOVED)
L13 12 DUP REM L11 (11 DUPLICATES REMOVED)

=> d bib 1-3 112

L12 ANSWER 1 OF 3 CAPLUS COPYRIGHT 2000 ACS
AN 1997:265594 CAPLUS
DN 126:247559
TI Rodent **whey acid protein (WAP)** or
mouse mammary tumor virus (MMTV) regulatory sequences for targeted expression of heterologous
genes in **human mammary cells** and
applications in carcinoma gene therapy
IN Guenzburg, Walter H.; Saller, Robert Michael; Salmons, Brian
PA Bavarian Nordic Research Institute A/s, Den.; Gsf - Forschungszentrum
Fuer Umwelt Und Gesundheit; Guenzburg, Walter H.; Saller, Robert Michael;
Salmons, Brian
SO PCT Int. Appl., 46 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9709440	A1	19970313	WO 1996-EP3922	19960906
	W: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
	RW: KE, LS, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM				
	AU 9669876	A1	19970327	AU 1996-69876	19960906
	EP 848757	A1	19980624	EP 1996-931040	19960906
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI				
	JP 11511979	T2	19991019	JP 1996-510876	19960906
PRAI	DK 1995-976		19950906		

L12 ANSWER 2 OF 3 CAPLUS COPYRIGHT 2000 ACS
 AN 1989:472068 CAPLUS
 DN 111:72068
 TI Thymic atrophy characteristic in transgenic mice that harbor pX genes of human T-cell leukemia virus type I
 AU Furuta, Yasuhide; Aizawa, Shinichi; Suda, Yoko; Ikawa, Yoji; Kishimoto, Hidehiro; Asano, Yoshihiro; Tada, Tomio; Hikikoshi, Atsuko; Yoshida, Mitsuaki; Seiki, Motoharu
 CS Tsukuba Life Sci. Cent., Phys. Chem. Inst., Tsukuba, 305, Japan
 SO J. Virol. (1989), 63(7), 3185-9
 CODEN: JOVIAM; ISSN: 0022-538X
 DT Journal
 LA English

L12 ANSWER 3 OF 3 BIOSIS COPYRIGHT 2000 BIOSIS
 AN 1979:175136 BIOSIS
 DN BA67:55136
 TI ISOLATION OF HOST RANGE VARIANTS OF **MOUSE MAMMARY TUMOR VIRUSES** THAT EFFICIENTLY INFECT CELLS IN-VITRO.
 AU HOWARDM D K; SCHLOM J
 CS LAB. VIRAL CARCINOGEN., NATL. CANCER INST., ROOM 1B19, BUILD. 37, BETHESDA, MD. 20014, USA.
 SO PROC NATL ACAD SCI U S A, (1978) 75 (11), 5718-5722.
 CODEN: PNASA6. ISSN: 0027-8424.
 FS BA; OLD
 LA English

=> d 1-12 au ti so l12

L12 ANSWER 1 OF 3 CAPLUS COPYRIGHT 2000 ACS
 IN Guenzburg, Walter H.; Saller, Robert Michael; Salmons, Brian
 TI Rodent **whey acid protein (WAP)** or **mouse mammary tumor virus (MMTV)** regulatory sequences for targeted expression of heterologous genes in **human mammary cells** and applications in carcinoma gene therapy
 SO PCT Int. Appl., 46 pp.
 CODEN: PIXXD2

L12 ANSWER 2 OF 3 CAPLUS COPYRIGHT 2000 ACS
 AU Furuta, Yasuhide; Aizawa, Shinichi; Suda, Yoko; Ikawa, Yoji; Kishimoto, Hidehiro; Asano, Yoshihiro; Tada, Tomio; Hikikoshi, Atsuko; Yoshida, Mitsuaki; Seiki, Motoharu
 TI Thymic atrophy characteristic in transgenic mice that harbor pX genes of human T-cell leukemia virus type I
 SO J. Virol. (1989), 63(7), 3185-9
 CODEN: JOVIAM; ISSN: 0022-538X

L12 ANSWER 3 OF 3 BIOSIS COPYRIGHT 2000 BIOSIS
 AU HOWARDM D K; SCHLOM J
 TI ISOLATION OF HOST RANGE VARIANTS OF **MOUSE MAMMARY TUMOR VIRUSES** THAT EFFICIENTLY INFECT CELLS IN-VITRO.
 SO PROC NATL ACAD SCI U S A, (1978) 75 (11), 5718-5722.
 CODEN: PNASA6. ISSN: 0027-8424.

=> s 1-12 au ti so l13

MISSING OPERATOR SO L13

The search profile that was entered contains terms or nested terms that are not separated by a logical operator.

=> d 1-12 au ti so l13

L13 ANSWER 1 OF 12 CAPLUS COPYRIGHT 2000 ACS
IN Gunzburg, Walter H.; Karle, Peter; Saller, Robert Michael
TI Cytochrome P450 encoding **retroviral vectors** and their
use as antitumor agents
SO PCT Int. Appl., 25 pp.
CODEN: PIXXD2

L13 ANSWER 2 OF 12 CAPLUS COPYRIGHT 2000 ACS
IN Guenzburg, Walter H.; Saller, Robert Michael; Salmons, Brian
TI Rodent **whey acid protein (WAP)** or
mouse mammary tumor virus (MMTV) regulatory sequences for targeted expression of heterologous
genes in **human mammary cells** and applications in
carcinoma gene therapy
SO PCT Int. Appl., 46 pp.
CODEN: PIXXD2

L13 ANSWER 3 OF 12 MEDLINE DUPLICATE 1
AU Mrochen S; Klein D; Nikol S; Smith J R; Salmons B; Gunzburg W H
TI Inducible expression of p21WAF-1/CIP-1/SDI-1 from a **promoter**
conversion **retroviral vector**.
SO JOURNAL OF MOLECULAR MEDICINE, (1997 Nov-Dec) 75 (11-12) 820-8.
Journal code: B8C. ISSN: 0946-2716.

L13 ANSWER 4 OF 12 MEDLINE DUPLICATE 2
AU Niermann G L; Buehring G C
TI Hormone regulation of bovine leukemia virus via the long terminal
repeat.
SO VIROLOGY, (1997 Dec 22) 239 (2) 249-58.
Journal code: XEA. ISSN: 0042-6822.

L13 ANSWER 5 OF 12 CAPLUS COPYRIGHT 2000 ACS
IN Guenzburg, Walter H.; Salmons, Brian
TI Viral and plasmid vectors encoding **mouse mammary**
tumor virus Naf repressor or Sag antigen for control of
viral infections or lymphocyte gene therapy
SO PCT Int. Appl., 44 pp.
CODEN: PIXXD2

L13 ANSWER 6 OF 12 CAPLUS COPYRIGHT 2000 ACS
IN Guenzburg, Walter H.; Winder, David; Saller, Robert Michael
TI Vectors carrying therapeutic genes encoding antimicrobial peptides for
gene therapy
SO PCT Int. Appl., 54 pp.
CODEN: PIXXD2

L13 ANSWER 7 OF 12 CAPLUS COPYRIGHT 2000 ACS
IN Guenzburg, Walter Henry; Saller, Robert Michael
TI Safe, non-self-inactivating retroviral expression vectors using non-LTR
promoters for gene therapy
SO PCT Int. Appl., 40 pp.
CODEN: PIXXD2

L13 ANSWER 8 OF 12 MEDLINE DUPLICATE 3
AU Wilson S E; Weng J; Blair S; He Y G; Lloyd S
TI Expression of E6/E7 or SV40 large T antigen-coding oncogenes in
human corneal endothelial cells indicates regulated
high-proliferative capacity.
SO INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE, (1995 Jan) 36 (1) 32-40.
Journal code: GWI. ISSN: 0146-0404.

L13 ANSWER 9 OF 12 BIOSIS COPYRIGHT 2000 BIOSIS
 AU Mellentin-Michelotti, Julia; John, Sam; Pennie, William D.; Williams, Trevor; Hager, Goprdon L. (1)
 TI The 5' **Enhancer** of the **Mouse Mammary Tumor Virus** Long Terminal Repeat Contains a Functional AP-2 Element.
 SO Journal of Biological Chemistry, (1994) Vol. 269, No. 50, pp. 31983-31990.
 ISSN: 0021-9258.

L13 ANSWER 10 OF 12 MEDLINE DUPLICATE 4
 AU Furuta Y; Aizawa S; Suda Y; Ikawa Y; Kishimoto H; Asano Y; Tada T; Hikikoshi A; Yoshida M; Seiki M
 TI Thymic atrophy characteristic in transgenic mice that harbor pX genes of **human T-cell leukemia virus type I**.
 SO JOURNAL OF VIROLOGY, (1989 Jul) 63 (7) 3185-9.
 Journal code: KCV. ISSN: 0022-538X.

L13 ANSWER 11 OF 12 CAPLUS COPYRIGHT 2000 ACS
 AU Sherman, Levana; Gazit, Arnona; Yaniv, Abraham; Dahlberg, John E.; Tronick, Steven R.
 TI Nucleotide sequence analysis of the long terminal repeat of integrated caprine arthritis encephalitis virus
 SO Virus Res. (1986), 5(2-3), 145-55
 CODEN: VIREDF; ISSN: 0168-1702

L13 ANSWER 12 OF 12 CAPLUS COPYRIGHT 2000 ACS DUPLICATE 5
 AU Howard, David K.; Schlom, Jeffrey
 TI Isolation of host-range variants of **mouse mammary tumor viruses** that efficiently infect cells in vitro
 SO Proc. Natl. Acad. Sci. U. S. A. (1978), 75(11), 5718-22
 CODEN: PNASA6; ISSN: 0027-8424

=> d bib 1 2 4 5 6 113

L13 ANSWER 1 OF 12 CAPLUS COPYRIGHT 2000 ACS
 AN 1997:650467 CAPLUS
 DN 127:315589
 TI Cytochrome P450 encoding **retroviral vectors** and their use as antitumor agents
 IN Gunzburg, Walter H.; Karle, Peter; Saller, Robert Michael
 PA Bavarian Nordic Research Institute A/S, Den.; GSF-Forschungszentrum Fur Umwelt Und Gesundheit; Gunzburg, Walter H.; Karle, Peter; Saller, Robert Michael
 SO PCT Int. Appl., 25 pp.
 CODEN: PIXXD2
 DT Patent
 LA English
 FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9735994	A2	199711002	WO 1997-EP1585	19970327
	WO 9735994	A3	19971120		
	W:	AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM			
	RW:	GH, KE, LS, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG			
	CA 2250173	AA	199711002	CA 1997-2250173	19970327

AU 9723827 A1 19971017 AU 1997-23827 19970327
 AU 713382 B2 19991202
 EP 892852 A2 19990127 EP 1997-919307 19970327
 R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
 IE, SI, LT, LV, FI, RO
 NO 9804540 A 19980928 NO 1998-4540 19980928
 PRAI DK 1996-352 19960327
 WO 1997-EP1585 19970327

L13 ANSWER 2 OF 12 CAPLUS COPYRIGHT 2000 ACS

AN 1997:265594 CAPLUS

DN 126:247559

TI Rodent whey acid protein (WAP) or

mouse mammary tumor virus (

MMTV) regulatory sequences for targeted expression of heterologous
 genes in human mammary cells and applications in
 carcinoma gene therapy

IN Guenzburg, Walter H.; Saller, Robert Michael; Salmons, Brian

PA Bavarian Nordic Research Institute A/s, Den.; Gsf - Forschungszentrum

Fuer

Umwelt Und Gesundheit; Guenzburg, Walter H.; Saller, Robert Michael;
 Salmons, Brian

SO PCT Int. Appl., 46 pp.

CODEN: PIXXD2

DT Patent

LA English

FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9709440	A1	19970313	WO 1996-EP3922	19960906
	W:	AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM			
	RW:	KE, LS, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM			
	AU 9669876	A1	19970327	AU 1996-69876	19960906
	EP 848757	A1	19980624	EP 1996-931040	19960906
	R:	AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI			
	JP 11511979	T2	19991019	JP 1996-510876	19960906
PRAI	DK 1995-976		19950906		
	WO 1996-EP3922		19960906		

L13 ANSWER 4 OF 12 MEDLINE

DUPLICATE 2

AN 1998096329 MEDLINE

DN 98096329

TI Hormone regulation of bovine leukemia virus via the long terminal repeat.

AU Niermann G L; Buehring G C

CS School of Public Health, University of California, Berkeley 94720, USA.

SO VIROLOGY, (1997 Dec 22) 239 (2) 249-58.

Journal code: XEA. ISSN: 0042-6822.

CY United States

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals; Cancer Journals

EM 199804

EW 19980402

L13 ANSWER 5 OF 12 CAPLUS COPYRIGHT 2000 ACS

AN 1996:661119 CAPLUS

DN 125:294771

TI Viral and plasmid vectors encoding mouse mammary

tumor virus Naf repressor or Sag antigen for control of
viral infections or lymphocyte gene therapy

IN Guenzburg, Walter H.; Salmons, Brian
PA Bavarian Nordic Research Institute A/s, Den.; GSF-Forschungszentrum fuer
Umwelt und Gesundheit GmbH
SO PCT Int. Appl., 44 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9628564	A1	19960919	WO 1996-EP1002	19960308
	W: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ				
	RW: KE, LS, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG				
	AU 9651040	A1	19961002	AU 1996-51040	19960308
	EP 817859	A1	19980114	EP 1996-907399	19960308
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI				
	JP 11508441	T2	19990727	JP 1996-527260	19960308
PRAI	DK 1995-244		19950309		
	WO 1996-EP1002		19960308		

L13 ANSWER 6 OF 12 CAPLUS COPYRIGHT 2000 ACS

AN 1996:661120 CAPLUS

DN 125:294754

TI Vectors carrying therapeutic genes encoding antimicrobial peptides for
gene therapy

IN Guenzburg, Walter H.; Winder, David; Saller, Robert Michael

PA Bavarian Nordic, Den.; GSF-Forschungszentrum fuer Umwelt und Gesundheit
GmbH

SO PCT Int. Appl., 54 pp.

CODEN: PIXXD2

DT Patent

LA English

FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9628563	A1	19960919	WO 1996-EP1001	19960308
	W: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ				
	RW: KE, LS, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG				
	AU 9651039	A1	19961002	AU 1996-51039	19960308
	EP 817858	A1	19980114	EP 1996-907398	19960308
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI				
	JP 11503305	T2	19990326	JP 1996-527259	19960308
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	WO 1996-EP1001		19960308		

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DUPLICATE 3

AB PURPOSE. Human corneal endothelial cells are thought

to have limited capacity for proliferation. Little is known about the mechanisms that regulate the proliferation of these **cells**. The authors introduced oncogenes into **human** corneal endothelial **cells** to modulate proliferation. In addition, they sought to establish **cell** lines to facilitate study of **human** corneal endothelial **cells**. METHODS. Early-passage **human** corneal endothelial **cells** were transduced with disabled **retrovirus** (pLXSN16E6/E7) coding for the human papilloma virus type 16 transforming oncoproteins E6 and E7. Early-passage cells were

also

stably transfected by electroporation with the pMTV-D305 plasmid vector, in which SV40 large T antigen (SV40 LTag) mRNA expression is positively regulated by the **mouse mammary tumor virus promoter**. Expression of E6/E7 mRNA or SV40 LTag mRNA in cell lines was monitored with the polymerase chain reaction. SV40 LTag protein expression was detected by immunocytology and Western blot analysis. RESULTS. **Human** corneal endothelial **cells** were efficiently infected with disabled **retrovirus** coding for E6/E7, and seven strains of cells have continued active proliferation for more than 50 population doublings (PD) (< 8 control PD). E6/E7 mRNA was expressed by each cell strain. E6/E7 transformed cells proliferate

rapidly

and form a monolayer of cells with a high degree of contact inhibition. Transfection with pMTV-D305 is less efficient, and only a single strain was developed. pMTV-D305-transfected endothelial cells (dexamethasone induced) proliferated at a lower rate than E6/E7-transduced cells or

cells

transfected with a vector (pSV3neo) in which SV40 LTag is constitutively regulated. In the absence of dexamethasone, the proliferation of pMTV-D305-transfected cells was even slower, but cells continued to produce SV40 LTag mRNA and protein. The latter results indicated that

SV40

LTag mRNA continued to be synthesized at significant levels in pMTV-D305-transfected cells in the absence of the inducer dexamethasone. CONCLUSIONS. This study suggests that **human** corneal endothelial **cells** have a high capacity for proliferation. Thus, **cell** division is normally controlled in **human** corneal endothelial **cells** by poorly characterized, but efficient, mechanisms. Because the E6 and E7 proteins, as well as the SV40 large T antigen, specifically bind to and interfere with the activity of the retinoblastoma (RB) and

p53

tumor suppressor proteins, our results suggest that these proteins have critical roles in regulating the proliferation of **human** corneal endothelial **cells**.